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(54) Title: PHARMACEUTICAL PREPARATIONS FOR INHIBITING TUMOURS ASSOCIATED WITH PROSTATE ADENOCARCINOMA, STOMACH CANCER AND BREAST CANCER

(57) Abstract

The present invention provides pharmaceutical preparations for inhibiting *in vitro* and *in vivo* cancerous prostate, gastrointestinal and breast tumours. In one embodiment the pharmaceutical preparation includes prostatic inhibin peptide which may be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from prostate cancer. In another embodiment the pharmaceutical preparation includes a mixture of prostatic inhibin peptide and a cancer drug which may be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from, for example gastrointestinal cancer. The cancer drug of the latter mixture may be one selected from the group of drugs including mitomycin, idalubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin.

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In ational application No.
PCT/CA93/00252

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 13-21, 23-24 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has be en carried out and based on the alleged effects of the compound/composition

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

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3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 93/00252

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/24

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	AMERICAN UROLOGICAL ASSOCIATION 87TH ANNUAL MEETING, WASHINGTON, D.C., USA, MAY 10-14, 1992. J.UROL. 147 (4 SUPPL.) 1992, page 335A 'Human Prostatic Inhibin suppresses growth of Dunning R3327-G Tumor in-vivo and inhibits Tumor Cell Colony in-vitro' * see Abstract no. 489 * ---	1-24
X,Y, P	THE PROSTATE vol. 22, no. 3, 1993, pages 225 - 233 'Effect of Prostatic Inhibin Peptide (PIP) on Prostate Cancer Cell Growth in Vitro and in Vivo' see page 225, line 3 - line 6 see page 233, ref. no. 9 see page 232, line 7 - line 10 see page 232, line 32 - line 36 --- -/--	1-24

° Special categories of cited documents :[10]

- "A" document defining the general state of the art which is not considered to be of particular relevance
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IV. CERTIFICATION

1

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 AUGUST 1993	25. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	GERLI P.F.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y,P	NATURE vol. 360, no. 6402, pages 313 - 19 'Alpha-inhibin is a tumor-suppressor gene with gonadal specificity in mice' see page 316, right column, line 3 - page 319, right column, line 30 -----	1-24

PHARMACEUTICAL PREPARATIONS FOR INHIBITING TUMOURS ASSOCIATED WITH PROSTATE ADENOCARCINOMA, STOMACH CANCER AND BREAST CANCER

FIELD OF THE INVENTION

The present invention relates to pharmaceutical preparations for use as tumour suppressive agents for tumours arising from prostatic adenocarcinoma, stomach cancer, breast cancer and benign prostatic hyperplasia.

BACKGROUND OF THE INVENTION

The prostate gland, which is found exclusively in male mammals, produces several components of semen and blood and several regulatory peptides. The prostate gland comprises stroma and epithelium cells, the latter group consisting of columnar secretory cells and basal nonsecretory cells. A proliferation of these basal cells as well as stroma cells gives rise to benign prostatic hyperplasia (BPH) which is one common prostate disease. Another common prostate disease is prostatic adenocarcinoma (CaP) which is the most common of the fatal pathophysiological prostate cancers and involves a malignant transformation of epithelial cells in the peripheral region of the prostate gland. Prostatic adenocarcinoma and benign prostatic hyperplasia are two common prostate diseases which have a high rate of incidence in the aging human male population. Approximately one out of every four males above the age of 55 suffers from a prostate disease of some form or another. Prostate cancer is the second most common cause of cancer related death in elderly men with there being approximately 96,000 cases diagnosed and about 26,000 deaths reported annually in the United States.

Studies of the various substances synthesized and secreted by normal, benign and cancerous prostates carried out in order to gain an understanding of the pathogenesis of the various prostate diseases reveal that

certain of these substances may be used as immunohistochemical tumour markers in the diagnosis of prostate disease. The three predominant proteins or peptides secreted by a normal prostate gland are Prostatic Acid Phosphatase (PAP), Prostate Specific Antigen (PSA) and Prostatic Inhibin Peptide which is known by several abbreviations including PIP, inhibin and Propep. Hereinafter Propep will be used to designate this material.

Metabolic and immunohistochemical studies have shown that the prostate is a major source of Propep. Propep is involved in the feedback control of, and acts to suppress secretion of, circulating follicle stimulating hormone (FSH) both in-vitro and in-vivo in adult male rats. Propep acts both at the pituitary as well as at the prostate site since both are provided with receptor sites for Propep.

Both PSA and PAP have been studied as tumour markers in the detection of prostate disease but since both exhibit elevated levels in prostates having benign prostatic hyperplasia neither marker is specific and therefore they are of limited utility.

Recently, it has been shown that Propep concentrations in serum of patients with BHP or CaP are significantly higher than normal. The highest serum concentration of Propep observed in normal men is approximately 40 ng/ml., while in men with either BPH or CaP serum concentrations of Propep have been observed in the range from 300-400 ng/ml. Because there exists some overlap in the concentrations of Propep in subjects having normal prostates and patients exhibiting either BHP or CaP, serum levels in and of themselves are of little value.

A major therapy in the treatment of prostate cancer is androgen-ablation. While most patients respond initially to this treatment, its effectiveness decreases over time possibly because of the presence of a heter-

ogenous population of androgen-dependant and androgen-independent cells to begin with. In such a scenario, the androgen sensitive cells respond to the androgen treatment while any androgen insensitive cells present would continue to proliferate unabated.

Other forms of cancer which are currently exacting a heavy toll are breast cancer in women and cancer of the gastrointestinal tract. Currently, the use of various cancer drugs such as mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin form part of the therapy for treating such cancers. One drawback to such a therapeutic treatment is the presence of adverse side effects due to the drugs in the concentration ranges required for effective treatment.

Accordingly, it would be advantageous to find a more effective means of arresting the growth of prostate, breast and gastrointestinal cancer cells and tumours which can be used effectively against both androgen sensitive and androgen insensitive cells.

SUMMARY OF THE INVENTION

The present invention provides in one aspect a pharmaceutical preparation comprising a peptide selected from the group consisting of prostatic inhibin peptide and analogues of prostatic inhibin peptide in a predetermined concentration range.

In another aspect of the invention there is provided a pharmaceutical preparation for inhibiting in-vivo the growth of various cancerous tumour growths which comprises peptides from the group including prostatic inhibin peptide and synthetic analogues of prostatic inhibin peptide in a predetermined concentration range. In this aspect of the invention, the pharmaceutical preparation is directed to cancerous tumour growths in the prostate and includes a pharmaceutically acceptable carrier and wherein the

concentration of the prostate inhibin peptide is generally greater than the concentration of prostate inhibin peptide observed in diseased prostates.

In this same aspect of the invention, the pharmaceutical preparation directed to inhibiting the growth of cancerous tumours may further include a cancer drug, for example, a drug selected from the group of cancer drugs including mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin.

In another aspect of the invention, there is provided a pharmaceutical preparation for inhibiting in-vivo the growth of prostate cancer tumours which comprises peptides from the group including prostatic inhibin peptide and synthetic analogues of prostatic inhibin peptide in an appropriate pharmaceutical dosage form.

In still another aspect of the invention there is provided a method of medical treatment for treating patients with various cancers which comprises administering a pharmaceutical preparation in an appropriate pharmaceutical dosage form, the pharmaceutical preparation comprising peptides from the group including prostatic inhibin peptide and synthetic analogues of prostatic inhibin peptide in a predetermined concentration range. In this aspect of the invention the method is directed preferably to treatment of prostate cancer. Where the method is directed to treatment of cancer of the gastrointestinal tract, the pharmaceutical preparation additionally optionally includes a cancer drug, the cancer drug being from the group of cancer drugs including mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin, the mixture being in an appropriate pharmaceutical dosage form.

LIST OF TABLES

Table I summarizes data showing the effect of Propep administration on the serum levels of FSH and LH (ng/ml^{-1}) in intact adult male rats;

Table II summarizes data showing the effect of Propep on cell proliferation;

Table III summarizes data showing the effect of Propep on the weight (grams) of testes and prostate;

Table IV summarizes in-vivo data relating to Propep dosage levels and subsequent tumour viability; and

Table V summarizes data on various hormone levels measured in rats 14 days after treatment with two different levels of Propep.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described, reference being had to the drawings, in which:

Figure 1 shows the complete sequence of human Propep;

Figure 2 illustrates an HPLC profile of Propep on a gel permeation column (LKB-TSK G 3000 SW 7.5x600 mm), the material being eluted as a major peak;

Figure 3 illustrates a reverse phase HPLC of purified Propep on a column of Lichrosorb RP-18 (5μm;0.4x25 cm, eluant, A 0.1% (w/v) aqueous TFA; B, 50% CH_3CN in 0.1% aqueous TFA; Flow rate, 1 ml/min, inset: SDS gel electrophoresis pattern of purified Propep (method of Laemmli, 1970);

Figures 4-9 display plots summarizing various studies of the effect of Propep on the in-vitro cells and in-vivo cancerous tumours as described below;

Figure 10 summarizes studies of the effect of FSH on prostate cancer cell growth in-vitro and its inhibition by Propep;

Figures 11 and 12 illustrate the complete human sequence of human Propep with the various fragments shown enclosed in boxes having utility in the pharmaceutical

compositions of the present invention;

Figure 13 summarizes the data of Table IV in bar graph form; and

Figures 14-20 summarize studies of the effect of Propep, various cancer drugs and combinations of Propep with these various cancer drugs on in-vitro gastric cancer cells.

DESCRIPTION OF THE INVENTION

The inventors have considered that high levels of Propep under pathophysiological conditions associated with prostate cancer may serve as a form of defence mechanism, albeit apparently not always effective, which may be initiated by the prostate. Various in-vivo and in-vitro experimental studies have been carried out and are summarized herebelow to determine the efficacy of concentrations of Propep higher than concentrations secreted by the diseased prostate as tumour suppressive agents for arresting or inhibiting the growth of prostatic adenocarcinoma. Studies have also been carried out to determine the efficacy of synthetic analogues of Propep, specifically fragments having 10, 17 and 28 amino acid groups, as tumour suppressive agents. These synthetic analogues have been shown to closely mimic the action of Propep in suppressing circulating FSH levels preferentially without altering the levels of luteinizing hormone (LH).

The bar graph of Figure 10 summarizes studies of the effect of FSH on prostate cancer cell growth in-vitro and its inhibition by Propep. The tumour cells were exposed for 48 hours to Propep with 0.5% serum in tissue cultures.

PREPARATION OF PROPEP

Referring to Figure 1, Propep is a simple nonglycosylated protein comprising at least 94 amino acid residues. Native Propep is known to exist in two forms,

10.5 kd and 16 kd. The gene for Propep has been cloned from human prostate using recombinant DNA technology.

Propep antigen was purified according to the basic procedure of Thakur et al.(1981) ISOLATION AND PURIFICATION OF INHIBIN FROM HUMAN SEMINAL PLASMA, Indian Journal of Experimental Biology, 19, 307-313 but with modifications (Thakur et al. and Sheth et al. (1984) CHARACTERIZATION OF A POLYPETIDE FROM HUMAN SEMINAL PLASMA WITH INHIBIN (INHIBITION OF FSH SECRETION)-LIKE ACTIVITY, FEBS Letters, 165, 11-15.). Sperm-free human seminal plasma was precipitated with alcohol (1:4 vol/vol) and then extracted with 0.05 M acetate buffer, pH 4.0. The soluble proteins were separated using chromatography on a Sephadex G-100 column (3.5x100 cm) using 0.01 M acetate buffer for equilibrium and elution. The fraction with FSH-suppressing activity was subjected to ion-exchange chromatography on DEAE-cellulose (3x30 cm). The column was washed initially with 0.05 Tris buffer, pH 8.0. The bound material was eluted using an NaCl gradient (0-0.5 M) in the same buffer. The active material collected was subsequently purified by high pressure liquid chromatography (HPLC) using a gel permeation column (LKB-TSK G-3000 S.W., 7.5 x 600 mm) and 0.01 M acetate buffer, pH 4 for equilibration and elution, see Figure 2.

The HPLC purified material exhibited a single band on SDS-Gel electrophoresis at pH 8.3 (see inset of Figure 3). On reverse phase HPLC, the purified material eluted as a single homogeneous peak, see Figure 3.

The fractions obtained at each stage of purification were assayed for bioactivity using intact adult male rats. The assay is based on suppression of circulating FSH levels. Administration of HPLC-purified Propep to adult male rats for 3 consecutive days caused specific suppression of circulating FSH levels, see Table 1. No significant change in LH levels was observed.

SYNTHESIS OF DECAPEPTIDE PROPEP ANALOGUE

The decapeptide analogue of Propep forming part of the subject invention disclosed herein is a synthetic analogue of the 85-94 amino acid residues at the carboxy terminal of the Propep sequence. The decapeptide differs from Propep in that the lysine residue at position 85 in Propep is replaced by a tyrosine residue and the cysteine residue at position 87 is protected by an acetomidomethyl group. This synthetic decapeptide and other fragments were prepared using an Automated Peptide Synthesizer.

IN-VITRO AND IN-VIVO STUDIES

Studies were carried out using the rat Dunning R-3327-G tumour which is a pre-eminent animal model for the study of CaP. The Dunning tumour is a fast growing, poorly differentiated, transplantable tumour which can be maintained both <u>in-vivo</u> in the Copenhagen x Fisher 344 rat and <u>in-vitro</u> as a cell line.

<u>EXPERIMENT 1</u>

EFFECT OF PROPEP ON IN-VITRO CELLS

Dunning tumour R-3327-G lines derived from cells dissociated in their 20th and 28th <u>in-vivo</u> passages in Copenhagen x 344 male carrier rats were used for the in-vitro studies. Tumours were excised and dissociated into single cells and cultured in T-25 culture flasks (Corning N.Y). Dissociated tumour cells were dislodged from the culture flask by trypsinization (0.25% trypsin and 0.02% EDTA at 37°C for 3 minutes) and passaged in alpha-MEM (GIBCO Labs, Grand Island, N.Y.) supplemented with 2 mM L-glutamic acid, 20% fetal bovine serum (FBS, Hyclone Labs., Logan, V.T.) and antibiotics (complete medium = CM). Cultures were passaged every five days.

For colony assay, R-3327-G cells between 2 and 10 in-vitro passages were trypsinized, dispersed into single cell suspension and cultured in 35 mm tissue culture dishes at 0.5 - 1.0 x 10^1 viable cells in 2ml CM.

Propep was diluted at various concentrations in CM, filtered, sterilized and then added at appropriate concentration to culture dishes. These culture dishes were incubated in a humidified incubator at 37°C with 5% CO_2 for seven days. Following this the culture dishes were emptied, washed twice in cold phosphate buffer saline (PBS) solution and then fixed in absolute methanol for 5 minutes. The culture dishes were then stained with acidified Harris Hematoxylin and the colonies were counted manually.

Between about 20% to 30% of plated R-3327-G cells formed characteristic diffuse colonies within 7 days. Typically, colonies consisted of 102.3 ± 13.7 cells. A dose dependent inhibition of both colony number and colony size were observed with addition of various concentrations of Propep. Above concentrations of 100 ng/ml the colony inhibition was significant, leading to a 50% reduction at a Propep concentration of 1 μg/ml. Increasing concentrations of Propep resulted in small cell-clusters (50 cell-Figure 4). Replenishing the culture media along with the Propep on the 4th day of the culture resulted in more effective and consistent inhibition of colony growth than that of one time Propep addition.

<u>EXPERIMENT 2</u>

EFFECT OF DECAPEPTIDE AND OTHER FRAGMENTS ON IN-VITRO CELLS

The synthetic decapeptide shown in the box in Figure 11 has been shown to mimic the biological action of Propep and therefore its effect on the R-3327-G cells was studied. Referring to Figure 5, the decapeptide synthetic analogue of Propep has a similar inhibitory action on in-vitro R-3327-G cell culture. Specifically, a 50% colony count inhibition was observed with 50 ng/ml of the decapeptide leading to a maximum of 70% inhibition at 1 μg/ml. However, referring again to Figure 4, an

equimolar concentration of the native Propep was found to have a greater inhibiting effect compared to the decapeptide. Other synthetic fragments having 17 and 28, amino acid groups, see Figure 12, have demonstrated a similar efficacy for suppressing prostate tumour growths, data not shown.

EXPERIMENT 3

EFFECT OF PROPEP ON ANDROGEN DEPENDENT AND INDEPENDENT R-3327-G IN-VITRO CELL COLONIES

The R-3327-G tumours comprise both androgen sensitive and androgen insensitive cells. The effect of Propep on these two cell populations was studied in-vitro. Cells were dissociated from a R-3327-G tumour in its 20th in-vivo passage and were cultured in the presence or absence of steriods. For comparison, cells from the 28th in-vivo passage known to be largely androgen insensitive were cultured in the same way.

The results of the effect of various concentrations of Propep on the in-vivo cells is summarized in Figure 6. The effect of Propep was similar under all test conditions for both androgen sensitive and androgen insensitive cells. Although the actual number of colonies which appeared under each assay condition were different with these cells, the extent of Propep induced colony inhibition was comparable in all.

EXPERIMENT 4

INHIBITION OF CELL-GROWTH BY PROPEP

Colony inhibition might occur as a result of immediate cell death or due to delay in the cell cycle. In order to distinguish between these two routes of inhibition, the following experiment was conducted.

Aliquots of 0.5 x 10^1 cells were cultured in 24 well plates and incubated with various concentrations of Propep. Cell counts were taken on days 3 and 7. In control wells the number of cells increased 4-fold after

3 days and 28-fold after 7 days. At a dose of Propep of 1 μg/ml, no increase in cell number was observed on day 3 while only a 5-fold increase was observed on day 7, see Table II.

The results of this study were further corroborated by measuring DNA synthesis using 3H-thymidine. Specifically, R-3327-G cells were cultured in 24-well tissue culture plates (Costar, MA) in the presence or absence of Propep for six days. 3H-thymidine (68 Ci/mmole, ICN Ca) diluted in CM containing 10 μM thymidine (Sigma MO) was added to duplicate culture wells (0.5 μCi/ml). Plates were further incubated for 18 hrs. The amount of 3H-thymidine incorporated was estimated by precipitation with trichloro acetic acid, as described previously. Figure 7 shows patterns of 3H-thymidine incorporation in Propep-incubated cultures on day 7, as depicted by DNA synthesis. Cultures that received Propep in the amount of 1 μg/ml had incorporated by day 7 only about 20% of radioactivity as compared to that of the control. The inhibitory effect of the Propep was more pronounced on day 7 than on day 3.

EXPERIMENT 5

IN-VIVO EXPERIMENT

Copenhagen x Fisher 344 F hybrid male rats were ear-tagged and implanted with R3327-G cells ($1x10^1$ cells/animal in the 28th in-vivo passage) as described earlier. The animals weighed approximately 500 grams at the time of tumour implantation. A treatment regimen was initiated when tumour volume measured 0.2 to 0.5 cc.

Tumour bearing animals were divided into two groups of eight. One group comprising the control group, received saline injection while the other group received Propep dissolved in saline in the amount of 5 μg/kg subcutaneously every day.

The tumour volume was approximated by 3-dimensional measurement using the formula 0.5236 x length

x width x depth. The rats were sacrificed 24 days after tumour implantation as control tumours at that point in time started becoming necrotic. Accessory sex organs and tumours were excised from the rats and weighed.

Significantly reduced tumour growth was observed in animals treated with Propep as compared to that of the saline group. Referring to Figure 8, the difference between the tumour volume in the control group and the Propep treated group became increasingly pronounced with longer treatment. As tumours in the control group started to become necrotic on day 24, tumour and accessory sex organs were excised and weighed on this day. Mean tumour weight of the Propep treated group was 2.66 ± 0.48 g as compared to 6.44 ± 1.19 g for the saline treated control group. A 58% reduction in tumour weight was observed at the end of the experiment i.e. on the 24 day following tumour implantation or on the 10th day following administration of Propep as compared to the saline treated control group. No significant change was observed in testes weight and prostate weight in Propep treated group, see Table III.

EXPERIMENT 6

IN-VIVO EXPERIMENT

The tumour bearing animals were divided into three group of 8 animals. The first group was the control group and received saline treatment. The second group received Propep in the amount of 5μg/kg and the third group received 1000 μg Leuprolide/kg. This treatment regimen continued until the tumour volume for each animal reached approximately 10 cc. Tumour volumes were measured twice a week as described earlier.

The tumour volume data for each individual tumour was log transformed. Statistical analysis between treated and control group was performed by student "t" test.

As these results clearly demonstrated a growth

inhibition following administration of Propep, the study was further extended to estimate tumour growth delay in Propep treated animals. Most of the tumours become necrotic by the time they reach 10 cc volume, following which the measurements may not be accurate thus, keeping this in mind, 10 cc was taken as an end point in this study. Among 8 animals in the treated group, tumour volume in 6 reached 10 cc by day 42 and in 2 by day 38. In the saline control group, tumour volume reached this size by day 30, see Figure 9. In other words, a delay of 10 days in tumour growth was observed in the Propep treated animals. In all experiments the difference in tumour growth rate curves of treated and control groups of animals was similar.

The cells used for the foregoing experiment were from the 28th in-vivo passage, which is a poorly differentiated androgen-insensitive tumour. In order to confirm this earlier observation, one group of animals were treated with Leuprolide which is an anti-androgen. There was no significant difference in the tumour growth rate of Leuprolide treated animals as compared to the saline control group.

EXPERIMENT 7

IN-VIVO EXPERIMENTS USING MAT-LYLU CELL LINES

The androgen independent Dunning rat adenocarcinoma cell lines, Mat-Lylu were obtained from Dr. J. T. Isaacs, Johns Hopkins Medical School, Baltimore, Maryland and cultured in the laboratory by using RPMI 1640 medium containing 10% fetal calf serum and 1% antibiotics. When the cells reached confluency, they were trypsinized, dispersed into single cell suspension and the cell count was taken using hemocytometer.

Tumours were induced in adult Copenhagen male rats weighing about 200 gms by subcutaneous injection of $2X10^6$ cells on two sides of the flank area. Animals were

segregated into different groups and the Propep injections were initiated on day 4 following the induction of tumour growth. Table IV shows the various concentrations of Propep injected into the animals.

Animals were injected every day and sacrificed on day 14 following the administration of tumour cells. The body weight and the tumour weights were recorded for both control and treated groups. Blood was collected through a cardiac puncture and serum FSH, LH, prolactin, testosterone and Propep were measured by radio immunoassay. These serum levels of the above mentioned hormones are summarized in Table V for the control group and animals treated with dosages of 5 ng and 50 ng of Propep. These results show that FSH levels decrease with dosage which suggests the mechanism of action of Propep relates to levels of FSH. In addition, testosterone levels are not adversely affected which indicates no loss of libido, in contrast to libido loss observed with current drugs used in the treatment of prostate and other forms of cancer.

A piece of tumour tissue from each animal was fixed in 10% buffered formalin to study the morphology of the cells. Table V shows the % viability of the tumours in treated groups when compared to the controls (100%). The results of table IV are summarized in the bar graph of Figure 13.

The foregoing studies show that propep, when administered in a predetermined concentration range, results in a significant inhibition, <u>in-vivo</u> of cancerous tumours associated with the prostate. Specifically, the Dunning rat studies with Propep show that an effective drug dosage "window" of between about 5 ng to 500 ng per 200 grams body weight exists. These results have been corroborated by several studies.

Those skilled in the art will be aware of the methods of preparing pharmaceutically appropriate dosage forms for Propep as applied to humans. Those skilled in

the art will also appreciate that such dosages may be encapsulated in time release delivery systems comprising for example a liposome delivery system, polysaccharides exhibiting a slow release mechanism, salistic or other polymer implants or microspheres.

EXPERIMENT 9

STUDY OF THE EFFECT OF PROSTATE INHIBIN PEPTIDE ON FRESH GASTRIC TUMOUR CELLS IN-VITRO BY METHYL TETRAZOLIUM SALT (MTT) ARRAY

Gastric tumour specimens were collected from patients with stomach cancer undergoing gastrectomy at Tata Memorial Hospital. Tumour specimens were collected in sterile DMEM and immediately transferred to the laboratory under cold conditions. The gastric tumour specimens were finely minced with a sterile pair of scissors. The finely minced gastric tissue was incubated with 1% collagenase 1 and IV in Dulbeco's minimum essential medium (DMEM) with 10% fetal calf serum (FCS) at 37°C with 5% CO_2 in an incubator for 1 hr. The whole mixture was then passed through a Millipore filter assembly and wire mesh (30 μm size) to get a single cell suspension of gastric tumour cells. The cells obtained were further subjected to primary culture in sterile culture bottles in 50 ml DMEM with 10% FCS and incubated for 12-18 hr. at 37°C with 5% CO_2 in an incubator, with 10 μl of 0.1, 0.5, 1.0, and 5.0 μg/ml concentration of Propep in a sterile 96 well microtitre plate. Blank and control in 6 microwells each were run along with tests. The plate was further incubated for 48 hrs. at 37°C in 5% CO_2. After 48 hrs., 10 μl of 5 mg/ml MTT was added in each well. After 6 hrs. of incubation at 37°C, 100 μl of 1 N HCl: Isopropanol (1:25) was added to each well and mixed vigorously to dissolve the farmazan crystals. Absorbance values at 540 nm were determined on an ELISA reader. Blank values were subtracted from the control and test values.

The percentage cell survival for each concentration of Propep along with concentrations of known 1) cancer drugs used in the treatment of gastric cancer including cisplatin, 5-fluoro-uracil, methotrexate, mitomycin, and 2) other cancer drugs used in chemotherapy including idarubicin, adriamycin, doxorubicin, and donomycin and combinations of Propep and these cancer drugs were calculated and compared to control. The results of these studies are summarized in Figures 14 to 20. As these results show, Propep by itself acts as a cytotoxin for stomach cancer cells. However, Propep used in combination with the various cancer drugs gives rise to a significantly enhanced cytoxic effect on cancerous cells as observed from Figures 14-20. The symbiotic effect obtained with the various combinations is evidenced by comparison to the pure Propep and cancer drugs. There is a further advantage obtained in addition to the significant beneficial, symbiotic effect in retarding cancer cell growth and proliferation obtained with the disclosed combinations of Propep and various cancer drugs. Specifically, since a significantly increased therapeutic effect is obtained with the above disclosed combinations utilizing lower concentrations of the cancer drugs compared to the treatment regimes in which the drugs are used alone, there is the potential to provide treatment wherein adverse side effects associated with the cancer drugs are considerably reduced than normally observed with the cancer drugs used alone in larger dosages.

The applicability of Propep, the fragments of Propep demonstrating an efficacy for inhibiting tumour growth and combinations of Propep with known cancer drugs for the treatment of various cancers found in mammals such as prostate cancer, breast and gastrointestinal cancer will be readily apparent to those skilled in the art. Further, the use of Propep and suitable fragments thereof for treatment of benign prostate hyperplasia will

also be apparent to those skilled in the art. The studies disclosed herein are interpreted to mean that Propep, the analogues thereof and combinations of Propep and the analogues with various cancer drugs will exhibit an efficacy in the treatment of diseases characterized by elevated levels of FSH in the body.

Various amounts of Propep in the range of 10 - 50 μg have been administered to adult male rats for a period of 4 to 12 weeks with no adverse toxic effect on body weight, or in parameters measured by clinical chemistry.

Those skilled in the art will be aware of pharmaceutically appropriate dosage forms for the mixtures of Propep and the cancer drugs as well as the manner in which a suitable dosage quantity and regimen may be derived in respect of a particular patient suffering from cancer of the gastrointestinal tract. In addition, those skilled in the art will also appreciate that such dosages may be encapsulated in time release delivery systems comprising for example a liposome delivery system, polysaccharides exhibiting a slow release mechanism, salistic or other polymer implants or microspheres.

While Propep, various fragments associated therewith, and combinations of Propep and the fragments with cancer drugs has been disclosed herein as exhibiting an efficacy for the treatment of prostate cancer and cancer of the gastrointestinal tract, it will be appreciated by those skilled in the art that numerous variations exist with respect to therapeutically treating various cancers characterized by elevated FSH levels Propep, the fragments and combinations of these compounds with various cancer drugs without departing from the scope of the invention.

TABLE I

Effect of Propep administration on the serum levels of FSH and LH ($\mu g\ ml^{-1}$) in intact adult male rats.

	FSH				LH			
	Saline	100 ng	1 μg	10 μg	Saline	100 ng	1 μg	10 μg
Mean± SEM	349	267.4*	223.7*	132*	402.2	398	386	351
(n = 5)	±20.8	±10.9	±10.2	±12.1	±28.6	±15.6	±30.3	±21.2
%Suppression	--	19.4	32.6	60.2	--	2.4	5.1	11.5

*P<0.001, in comparison with saline control.

Propep was administered (s.c.) daily for 3 days, and blood collected 2 h after the last injection.

Table II

Effect of Prostatic Inhibin Peptide Propep on cell Proliferation

TREATMENT			CELL COUNT	
		3 DAYS		7 DAYS
	CELLS/WELL	INHIBITION(%)	CELLS/WELL	INHIBITION(%)
CONTROL	2150	0	1.44 X 10 4	0
10/μg	330	84	0.28 X 10 4	80
5/μg	1165	45	0.708 X 10 4	50
1/μg	2000	7	0.958 X 10 4	30

R-3327 (G) cells were seeded at a cell density 500 cells/well in 16 mm multiwell plates in MEM supplemented with 15% FBS. Different concentrations of prostatic inhibin peptide was added as indicated. One plate was counted on day 3 while other plate was supplemented with indicated amount of Propep and cell counts were carried out 7 days after initial addition of Propep. Percentage inhibition was calculated taking control as 100% Values are means of triplicate.

Table III

Effect of Propep on Weight of Testis and Prostate Weight (grams)

	Testis	Prostate
Saline Control	3.26 +/- 0.19	1.26 +/- 0.24
Propep Treated	3.56 +/- 0.31	1.11 +/- 0.21

TABLE IV

GROUPS PROPEP DOSAGE	% VIABILITY WHEN COMPARED TO CONTROLS
0-CONTROL	100%
5 picograms p	100%
50 pg p	100%
0.5 nano	85%
	(Mean from two expts.)
5 ng	68%
50 ng	63%
500 ng	64%
	(Mean from two expts.)
5 μg	70%

TABLE V

HORMONE LEVELS IN THE RATE CIRCULATION

ANIMALS TREATED DAYS 3 - 13. ANIMALS SACRIFICED ON DAY 14.

DOSE	% TUMOUR INHIBITION	FSH (NG/ML)	PROLACTIN (NG/ML)	LH (NG/ML)	TESTO. (NG/ML)	PIP (NG/ML)
CONT.	0	9.35+/- .92	415+/-194	.68+/- .24	1.4+/- .27	7.48+/- 0.5
5NG	32%	4.6+/- .95	273+/-93	.39+/- .03	2.0+/- .67	7.0+/- 1.3
50 NG PIP	39%	3.73+/- .36	245+/-70	.30+/- .04	1.1+/- .98	9.39+/- 1.0

THEREFORE WHAT IS CLAIMED IS:

1. A pharmaceutical preparation comprising a peptide selected from the group consisting of prostatic inhibin peptide and analogues of prostatic inhibin peptide in a predetermined concentration range.

2. The pharmaceutical preparation of claim 1 for inhibiting the growth of benign prostatic hyperplasia or adenocarcinoma wherein the preparation includes a pharmaceutically acceptable carrier, and wherein the concentration of the peptide is greater than the concentration of prostatic inhibin peptide observed in diseased prostates.

3. The pharmaceutical preparation of claim 2 wherein the pharmaceutically acceptable carrier includes a time release encapsulation system for encapsulating the peptide.

4. The pharmaceutical preparation of claim 3 wherein the time release encapsulation system comprises a liposome delivery system.

5. The pharmaceutical preparation of claim 3 wherein the time release encapsulation system comprises a polysaccharide exhibiting a slow release mechanism.

6. The pharmaceutical preparation of claim 2 wherein the adenocarcinoma is adenocarcinoma of the prostate.

7. The pharmaceutical preparation of claim 2 wherein the adenocarcinoma is adenocarcinoma of the gastrointestinal tract.

8. The pharmaceutical preparation of claim 7 including a cancer drug.

9. The pharmaceutical preparation of claim 8 including a pharmaceutically acceptable carrier.

10. The pharmaceutical preparation of claim 9 wherein the pharmaceutically acceptable carrier includes a time release encapsulation system for encapsulating the combination of the prostatic inhibin peptide and the cancer drug.

11. The pharmaceutical preparation of claim 10 wherein the time release encapsulation system comprises a liposome delivery system.

12. The pharmaceutical preparation of claim 10 wherein the time release encapsulation system comprises a polysaccharide exhibiting a slow release mechanism.

13. A method of medical treatment for treating patients with benign prostatic hyperplasia or adenocarcinoma, the method comprising administering a pharmaceutical preparation in an appropriate pharmaceutical dosage form, the pharmaceutical preparation comprising a peptide selected from the group consisting of prostatic inhibin peptide and analogues of prostatic inhibin peptide.

14. The method of claim 13 wherein the adenocarcinoma is adenocarcinoma of the prostate and wherein the concentration of the peptide in the pharmaceutical preparation is greater than the concentration of prostatic inhibin peptide naturally observed in diseased prostates.

15. The method of claim 13 wherein the adenocarcinoma is cancer of the gastrointestinal tract.

16. The method of claim 15 wherein the pharmaceutical preparation includes a cancer drug.

17. The method of claim 13 wherein the adenocarcinoma is breast cancer.

18. The method of claim 17 wherein the pharmaceutical preparation includes a cancer drug.

19. A method of medical treatment for treating patients with diseases characterized by elevated levels of FSH which comprises administering a pharmaceutical preparation in an appropriate pharmaceutical dosage form, the pharmaceutical preparation comprising a peptide selected from the group consisting of prostatic inhibin peptide and analogues of prostatic inhibin peptide.

20. A method of medical treatment according to claim 19 wherein said analogue of prostatic inhibin peptide is an analogue selected from the group consisting of the R-10, R-17 and R-28 analogues.

21. A method of medical treatment for treating patients with diseases characterized by elevated levels of FSH which comprises administering a pharmaceutical preparation in an appropriate pharmaceutical dosage form, the pharmaceutical preparation comprising an analogue of prostatic inhibin peptide selected from the group consisting of the R-10, R-17 and R-28 analogues.

22. The pharmaceutical preparation of claim 8 wherein the cancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin.

23. The method of claim 16 wherein the cancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin.

24. The method of claim 18 wherein the cancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluorouracil, methotrexate, adriamycin and donomycin.

NH_2-Ser	Cys	Tyr	Phe	Ile	Pro	Asn	Glu	Gly	Val
1				5					10
Pro	Gly	Asp	Ser	Thr	Arg	Lys	Cys	Met	Asp
				15					20
Leu	Lys	Gly	Asn	Lys	His	Pro	Ile	Asn	Ser
				25					30
Glu	Trp	Gln	Thr	Asp	Asn	Cys	Glu	Thr	Cys
				35					40
Thr	Cys	Tyr	Glu	Thr	Glu	Ile	Ser	Cys	Cys
				45					50
Thr	Leu	Val	Ser	Thr	Pro	Val	Gly	Tyr	Asp
				55					60
Lys	Asp	Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys
				65					70
Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val	Glu	Lys
				75					80
Lys	Asp	Pro	Lys	Lys	Thr	Cys	Ser	Val	Ser
				85					90
Glu	Trp	Gly	Ile-COOH						
			94						

FIGURE 1

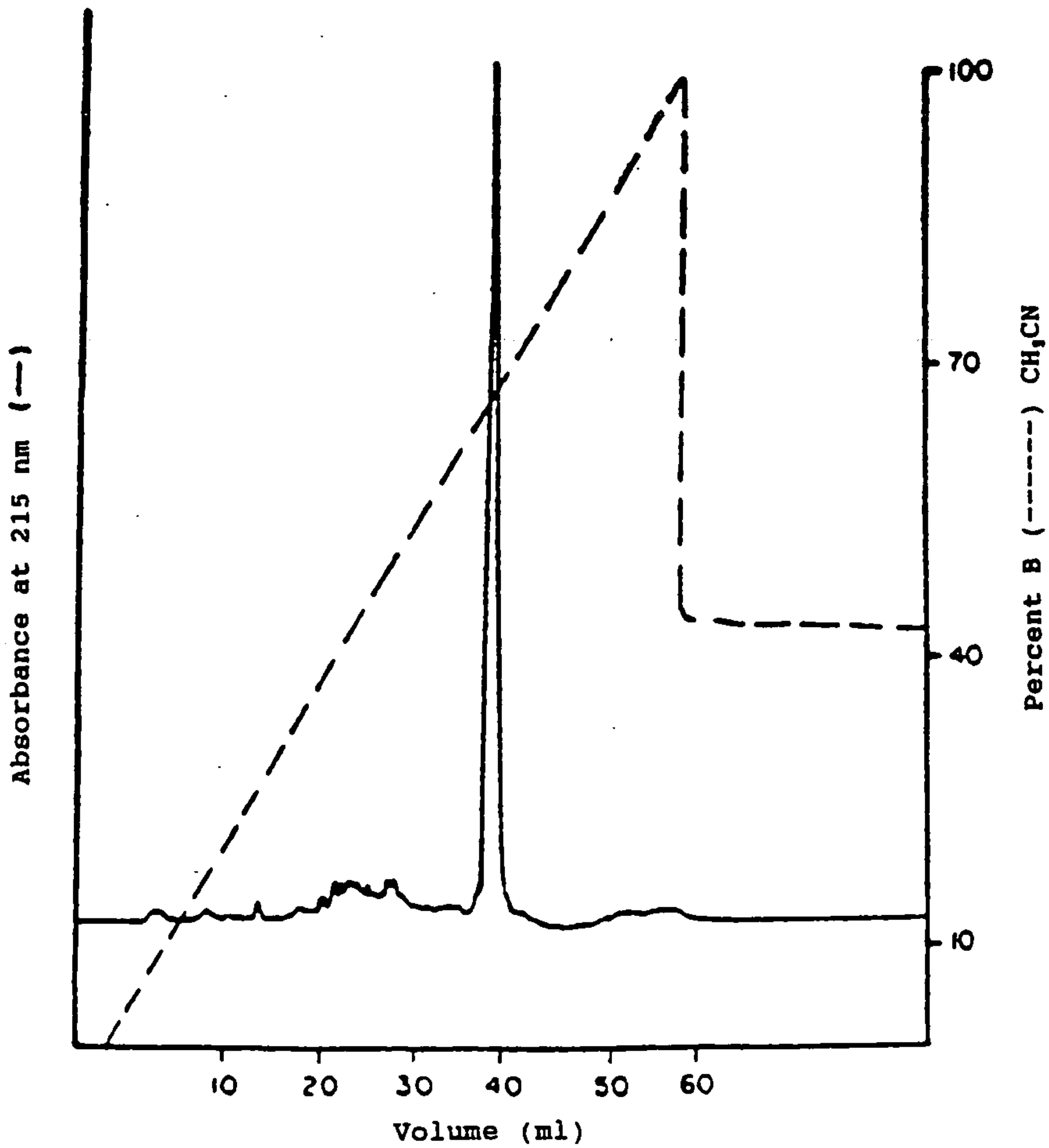

Absorbance at 215 nm (—)
Percent B (------) CH3CN
100
70
40
10
10
20
30
40
50
60
Volume (ml)

FIGURE 2

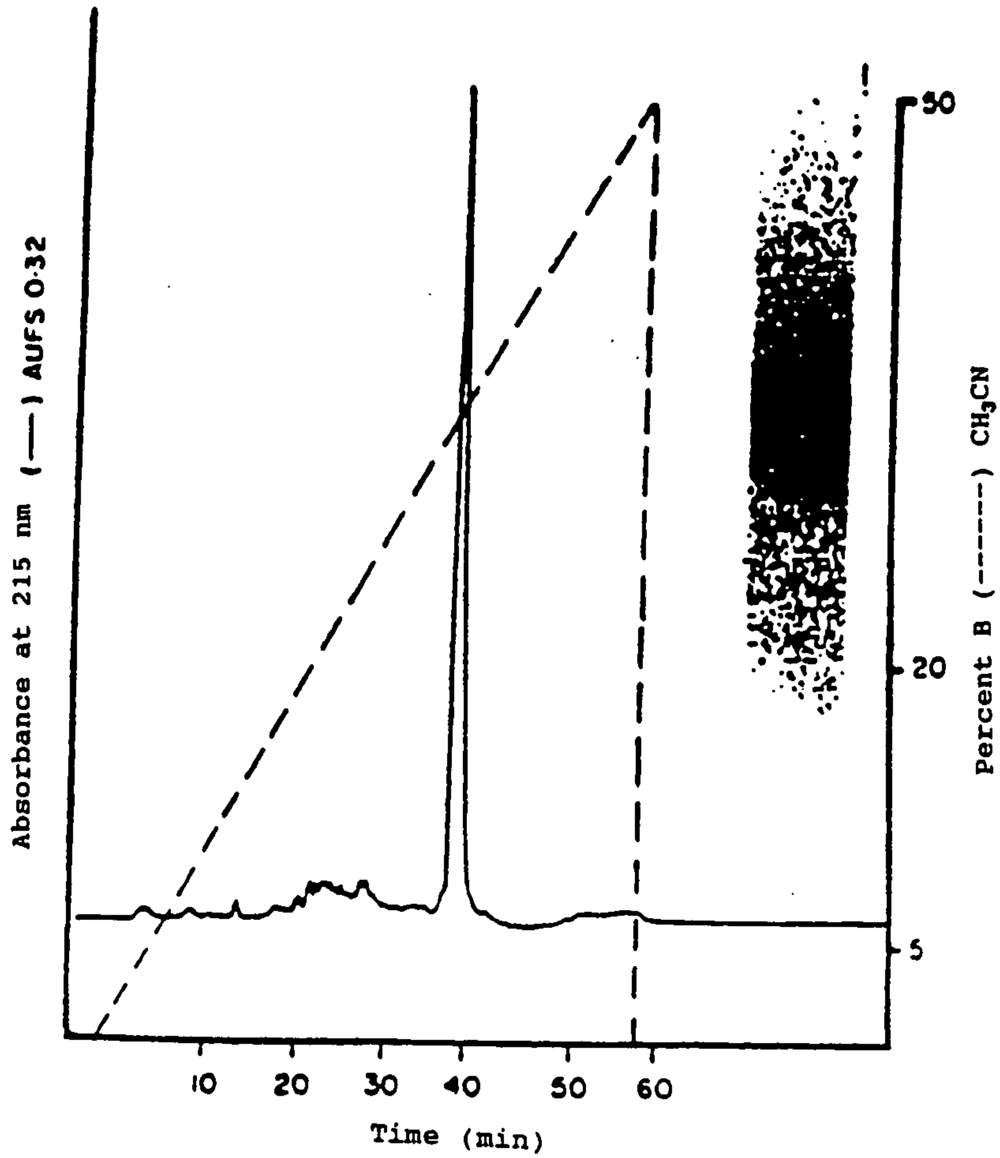
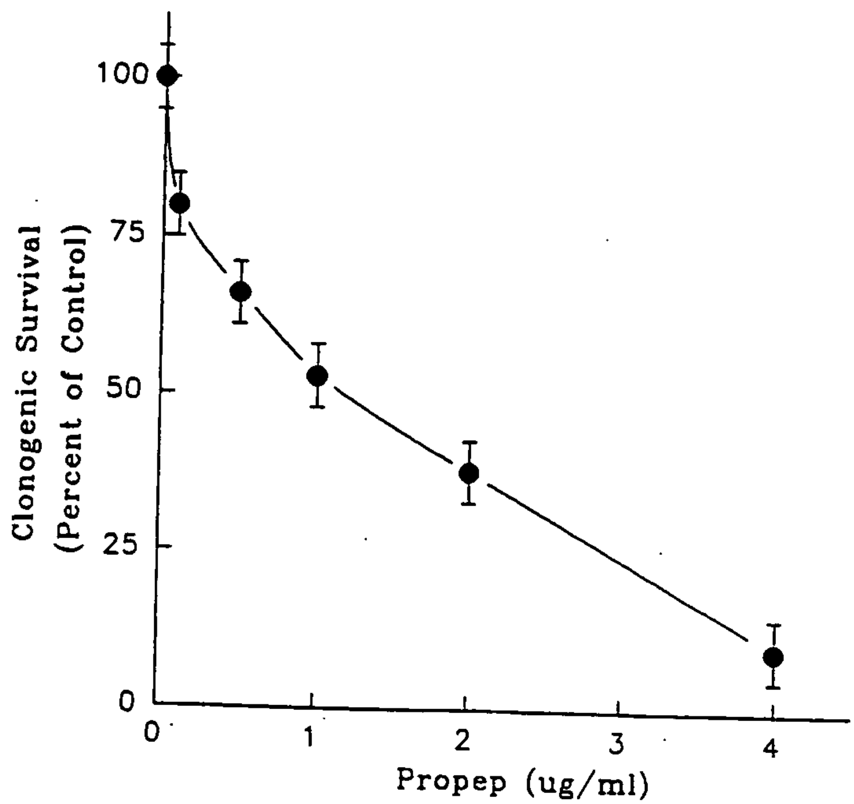
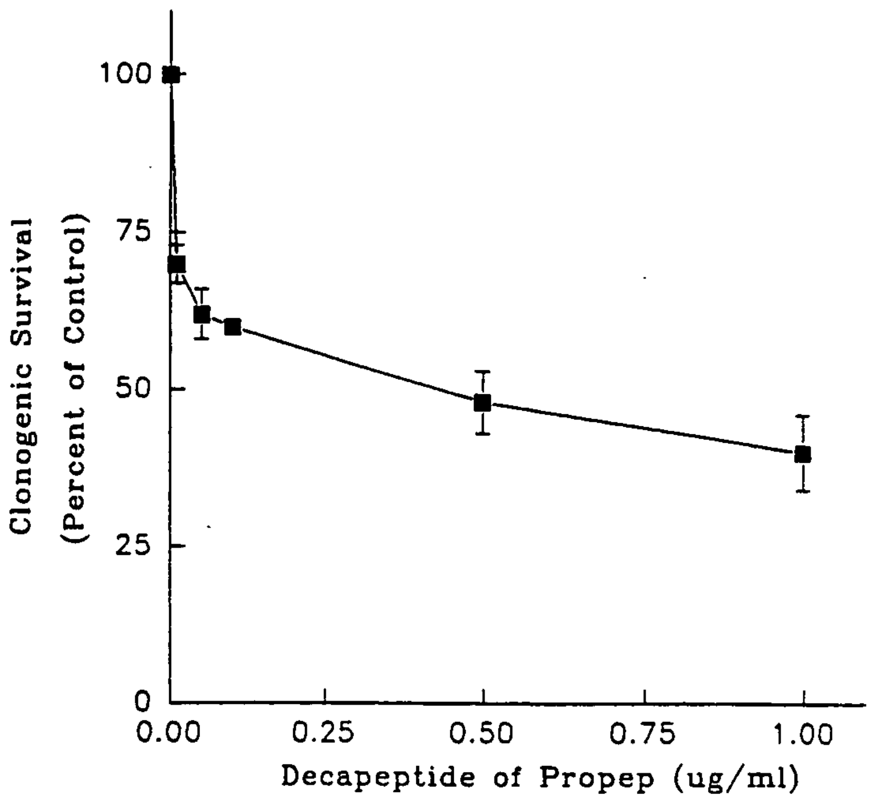

Absorbance at 215 nm (——) AUFS 0.32
Percent B (------) CH3CN
50
20
5
10
20
30
40
50
60
Time (min)

FIGURE 3



Clonogenic Survival
(Percent of Control)
100
75
50
25
0
0
1
2
3
4
Propep (ug/ml)

FIGURE 4



Clonogenic Survival
(Percent of Control)
100
75
50
25
0
0.00
0.25
0.50
0.75
1.00
Decapeptide of Propep (ug/ml)

FIGURE 5

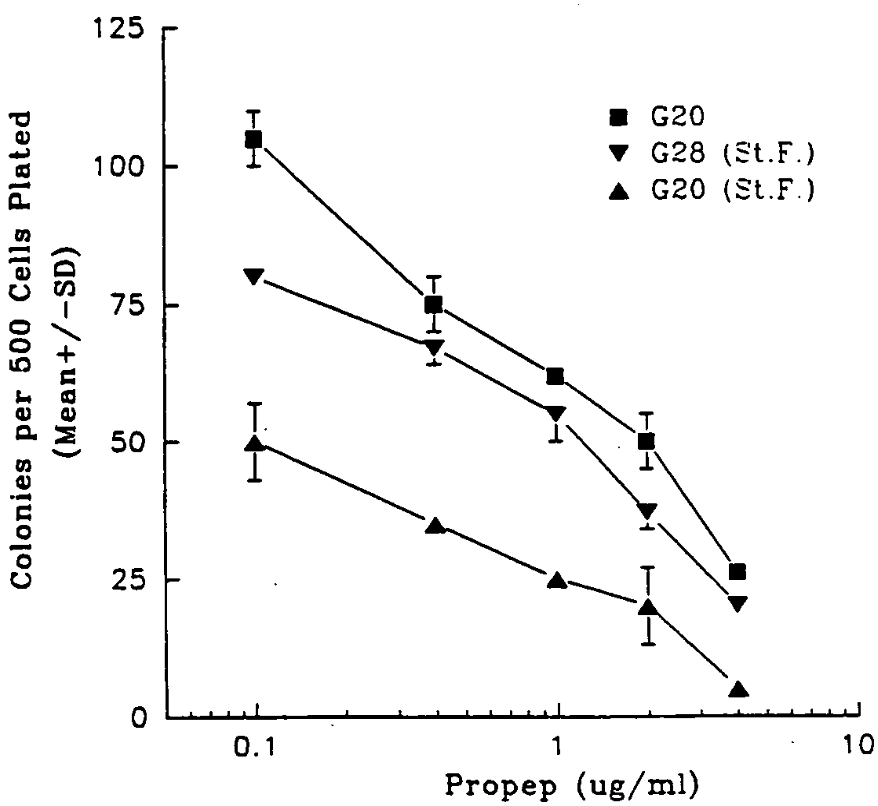

125
100
75
50
25
0
Colonies per 500 Cells Plated (Mean+/-SD)
G20
G28 (St.F.)
G20 (St.F.)
0.1
1
10
Propep (ug/ml)

FIGURE 6

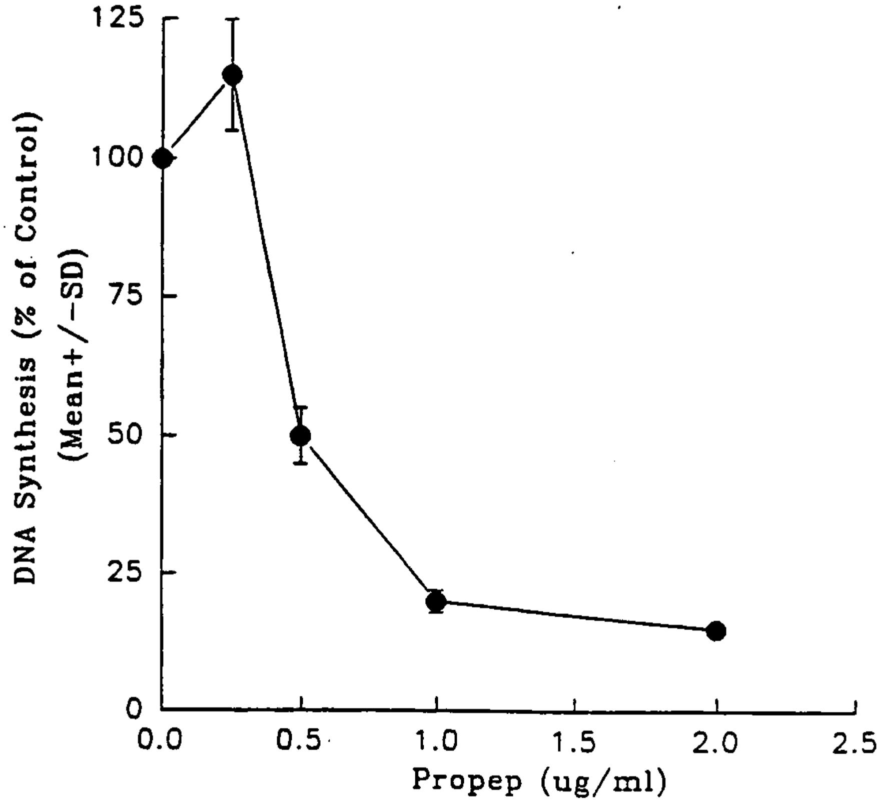

125
100
75
50
25
0
DNA Synthesis (% of Control)
(Mean+/-SD)
0.0
0.5
1.0
1.5
2.0
2.5
Propep (ug/ml)

FIGURE 7

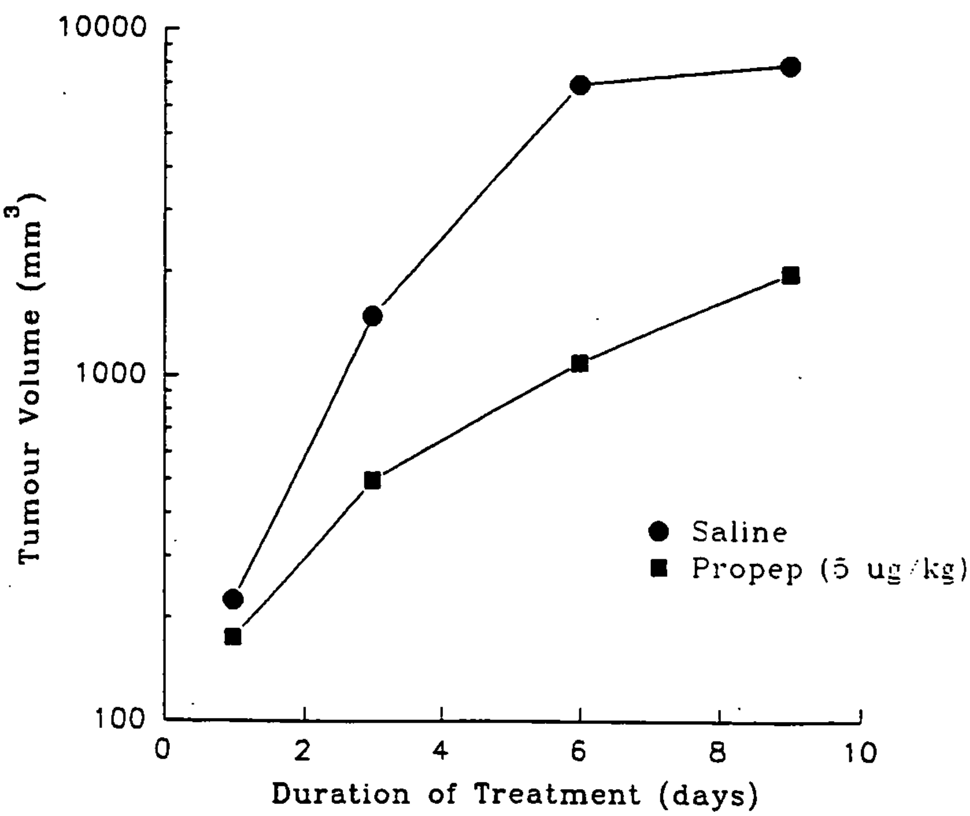

10000
1000
100
Tumour Volume (mm3)
0
2
4
6
8
10
Duration of Treatment (days)
Saline
Propep (5 ug/kg)

FIGURE 8

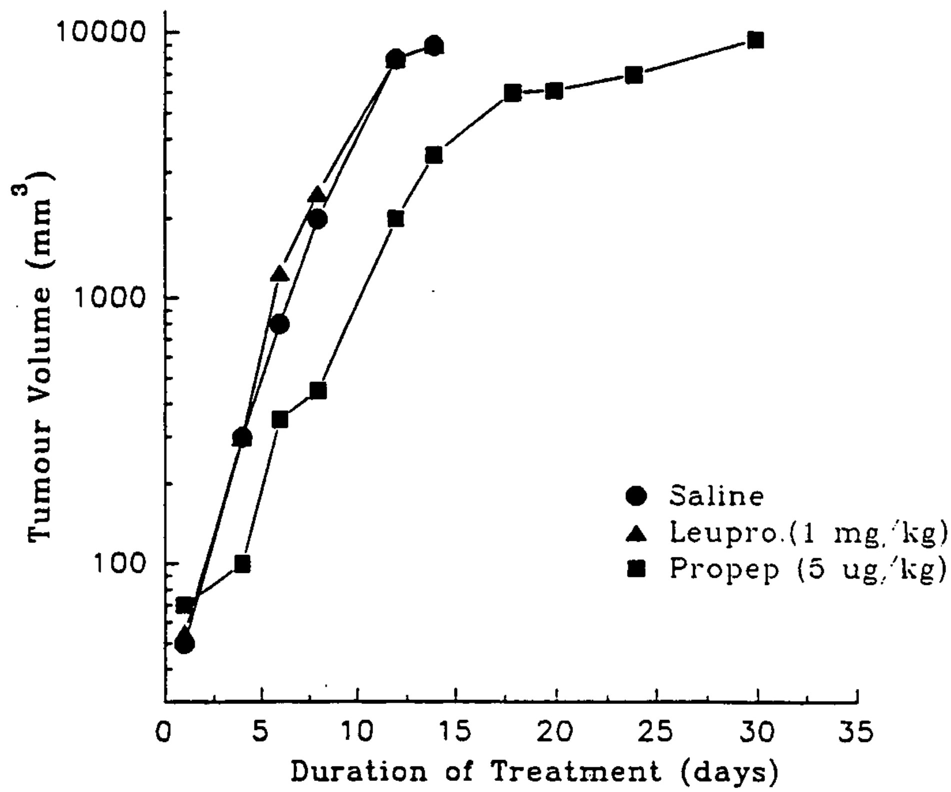


FIGURE 9

SUBSTITUTE SHEET

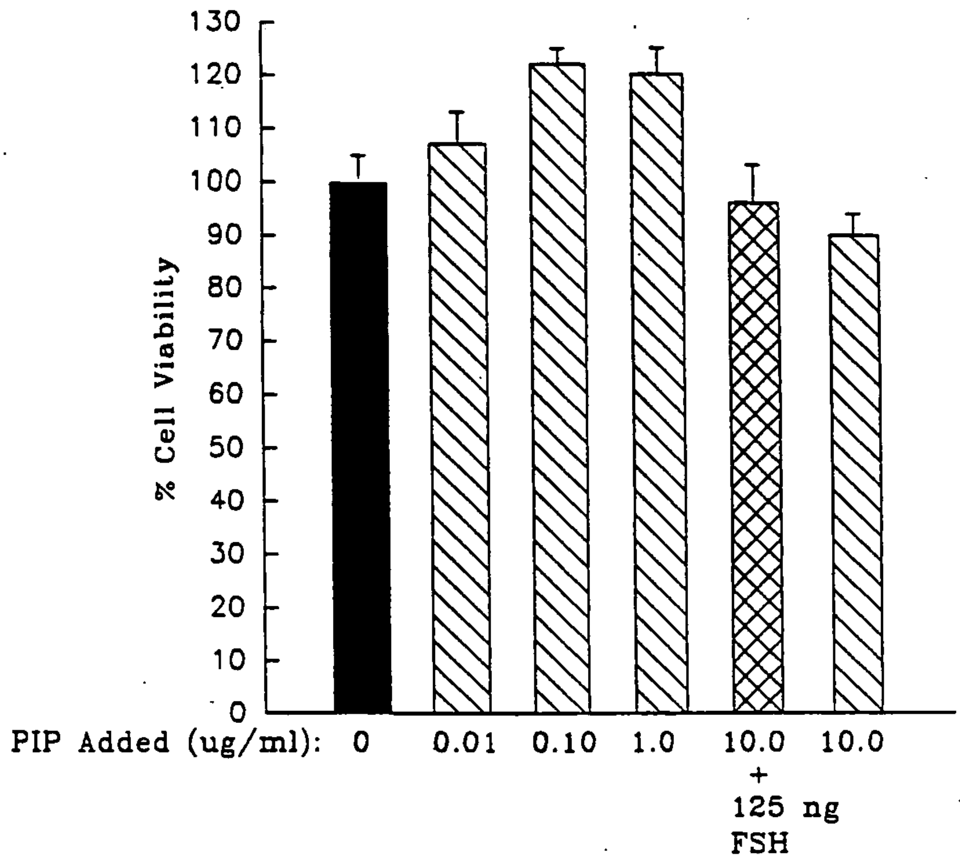

130
120
110
100
90
80
70
60
50
40
30
20
10
0
% Cell Viability
PIP Added (ug/ml): 0 0.01 0.10 1.0 10.0 + 125 ng FSH 10.0

FIGURE 10

NH_2-Ser	Cys	Tyr	Phe	Ile	Pro	Asn	Glu	Gly	Val
1				5					10
Pro	Gly	Asp	Ser	Thr	Arg	Lys	Cys	Met	Asp
				15					20
Leu	Lys	Gly	Asn	Lys	His	Pro	Ile	Asn	Ser
				25					30
Glu	Trp	Gln	Thr	Asp	Asn	Cys	Glu	Thr	Cys
				35					40
Thr	Cys	Tyr	Glu	Thr	Glu	Ile	Ser	Cys	Cys
				45					50
Thr	Leu	Val	Ser	Thr	Pro	Val	Gly	Tyr	Asp
				55					60
Lys	Asp	Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys
				65					70
Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val	Glu	Lys
				75					80
Lys	Asp	Pro	Lys	Lys	Thr	Cys	Ser	Val	Ser
				85					90
Glu	Trp	Gly	Ile-COOH						
			94						

R-10

FIGURE 11

R-17

NH_2-Ser	Cys	Tyr	Phe	Ile	Pro	Asn	Glu	Gly	Val
1				5					10
Pro	Gly	Asp	Ser	Thr	Arg	Lys	Cys	Met	Asp
				15					20
Leu	Lys	Gly	Asn	Lys	His	Pro	Ile	Asn	Ser
				25					30
Glu	Trp	Gln	Thr	Asp	Asn	Cys	Glu	Thr	Cys
				35					40
Thr	Cys	Tyr	Glu	Thr	Glu	Ile	Ser	Cys	Cys
				45					50
Thr	Leu	Val	Ser	Thr	Pro	Val	Gly	Tyr	Asp
				55					60
Lys	Asp	Asn	Cys	Gln	Arg	Ile	Phe	Lys	Lys
				65					70
Glu	Asp	Cys	Lys	Tyr	Ile	Val	Val	Glu	Lys
				75					80
Lys	Asp	Pro	Lys	Lys	Thr	Cys	Ser	Val	Ser
				85					90
Glu	Trp	Gly	Ile-COOH						
			94						

R-28

FIGURE 12

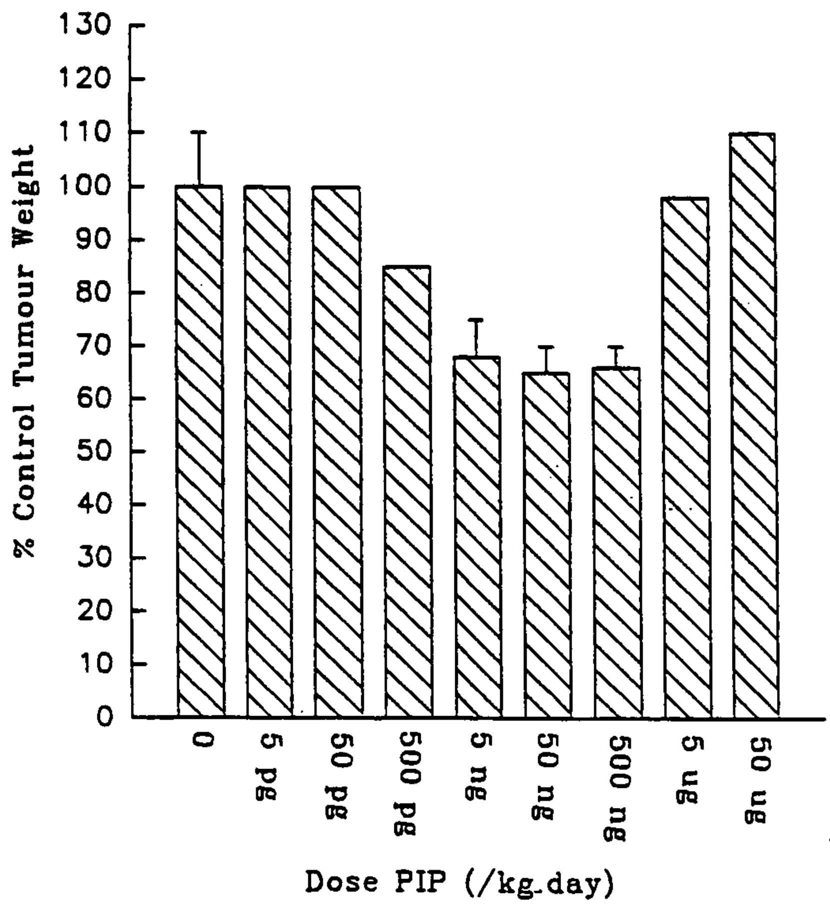
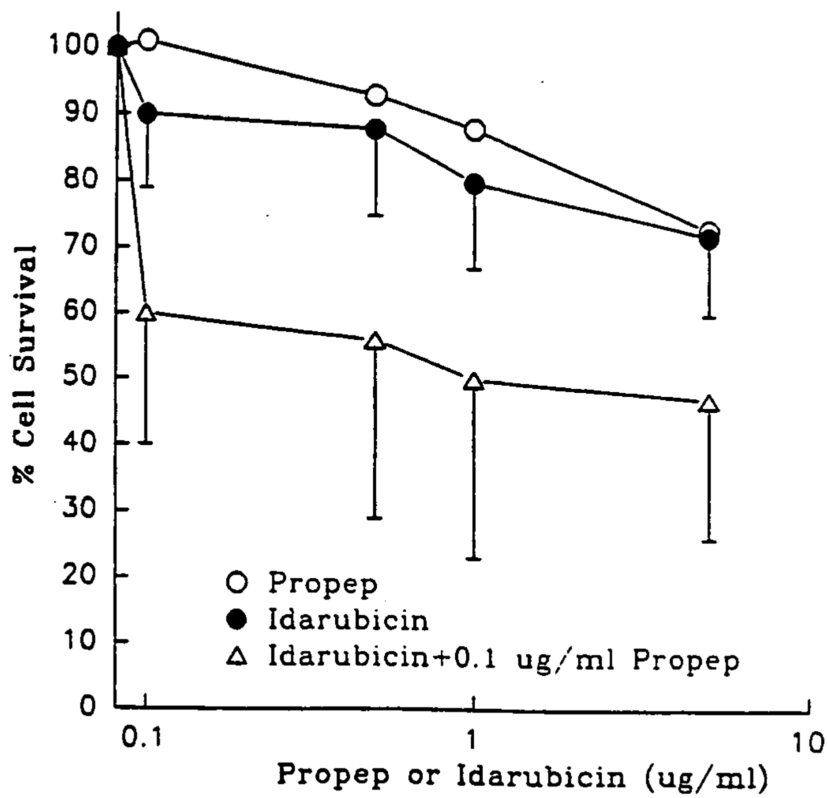

% Control Tumour Weight
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5 pg
50 pg
500 pg
5 ng
50 ng
500 ng
5 ug
50 ug
Dose PIP (/kg.day)

FIGURE 13



100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
Propep or Idarubicin (ug/ml)
Propep
Idarubicin
Idarubicin+0.1 ug/ml Propep

FIGURE 14

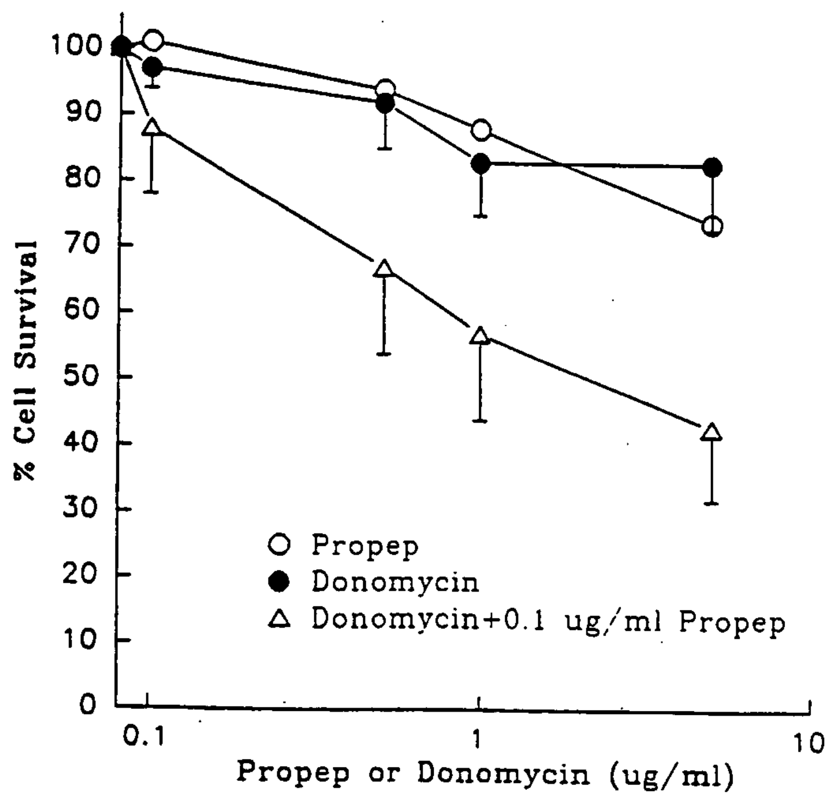
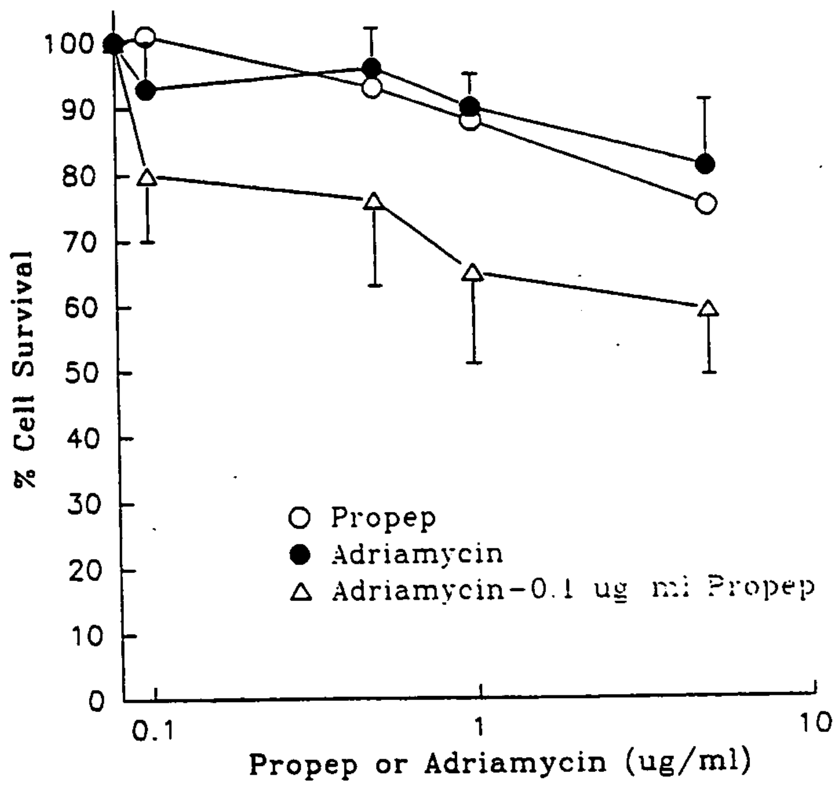
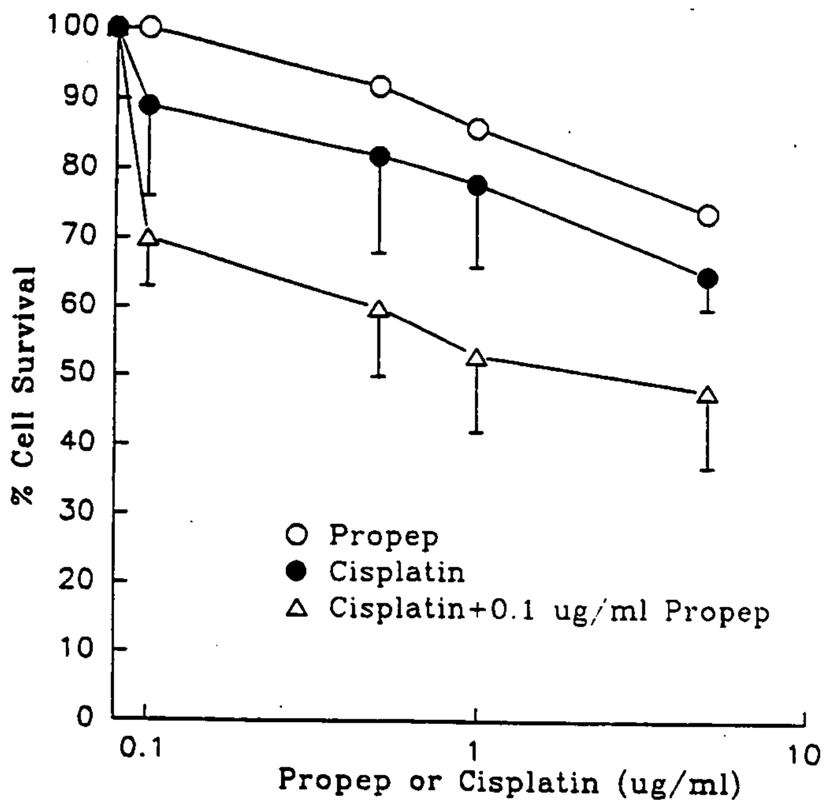

100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
Propep or Donomycin (ug/ml)
Propep
Donomycin
Donomycin+0.1 ug/ml Propep

FIGURE 15



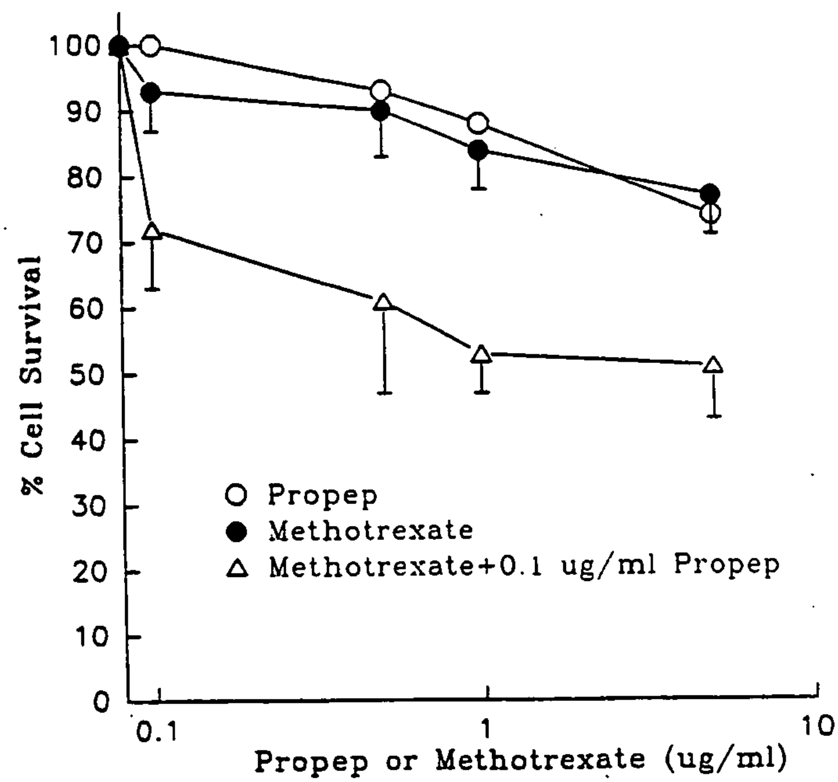
% Cell Survival
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
Propep or Adriamycin (ug/ml)
Propep
Adriamycin
Adriamycin-0.1 ug ml Propep

FIGURE 16



100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
Propep or Cisplatin (ug/ml)
Propep
Cisplatin
Cisplatin+0.1 ug/ml Propep

FIGURE 17



100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
0.1
1
10
Propep or Methotrexate (ug/ml)
Propep
Methotrexate
Methotrexate+0.1 ug/ml Propep

FIGURE 18

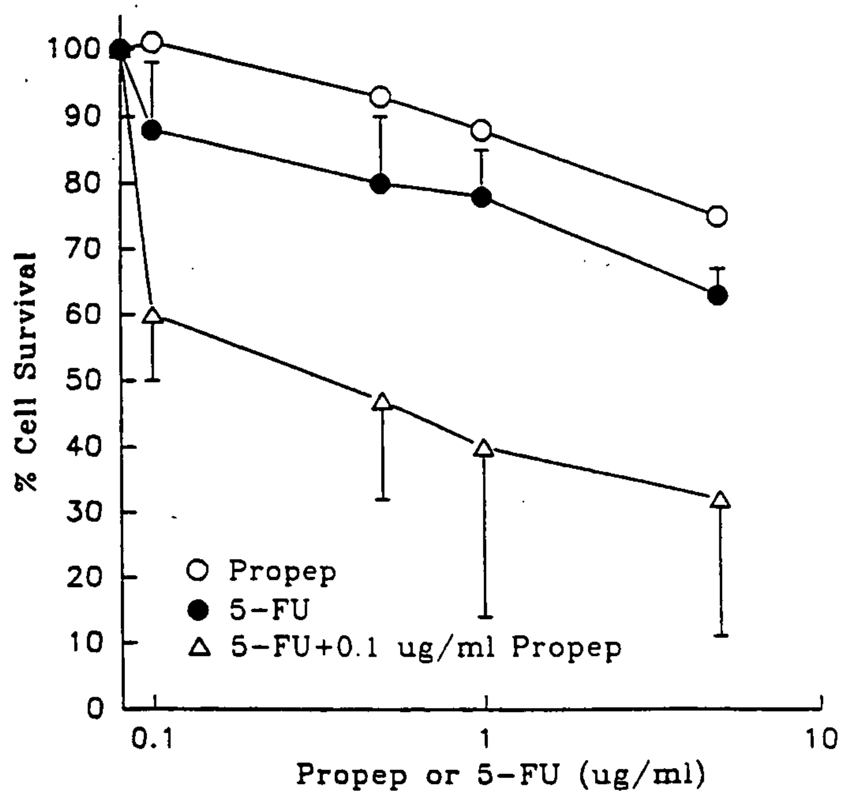

100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
Propep
5-FU
5-FU+0.1 ug/ml Propep
0.1
1
10
Propep or 5-FU (ug/ml)

FIGURE 19

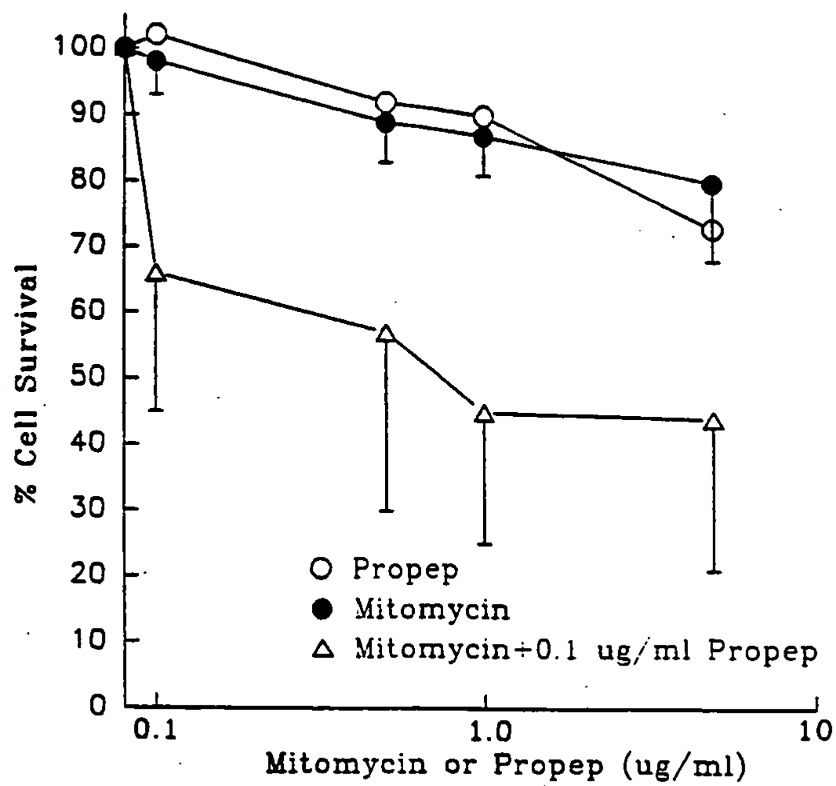

100
90
80
70
60
50
40
30
20
10
0
% Cell Survival
Propep
Mitomycin
Mitomycin+0.1 ug/ml Propep
0.1
1.0
10
Mitomycin or Propep (ug/ml)

FIGURE 20